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Embryonic Stem Cell Development in a Chemically Defined Medium

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Vertebrate germ layer development is an intricately interwoven process with the organism operating as an integrated whole. To examine these processes we have used embryonic stem (ES) cell *in vitro* differentiation in a serum-free, chemically defined medium (CDM). In CDM, ES cells differentiate as embryoid bodies to neuroectoderm with upregulation of pax-6, without commensurate expression of Brachyury. In the presence of Activin A, pax-6 and Brachyury mRNAs are readily detectable, suggestive of both neuroectoderm and mesoderm formation, while in the presence of BMP-4 a process resembling primitive streak formation at the molecular level occurs. Neuroectoderm development in CDM alone is consistent with the view that this process can occur by default, as reported in *Xenopus,* due to the absence or sequestration of mesoderm-inducing factors. Additionally, these data show that BMP-4 alone is capable of instigating a process resembling primitive streak formation in ES cells and possibly *in vivo*. © 1999 Academic Press

INTRODUCTION

The influences which mediate vertebrate mesoderm and neuroectoderm development/patterning are part of a dynamic complex interacting system. Pioneering studies using a combination of *in vitro* and *in vivo* assays with *Xenopus laevis* have shown that a number of defined polypeptide factors can influence primary germ layer formation in vertebrates (see, for example, [1, 2] and references therein). Similar experiments in mammals have, however, not been possible.

We have used embryonic stem (ES) cell *in vitro* differentiation as an approach to gaining a greater understanding of early mammalian development. ES cells resemble the inner cell mass (ICM) of the day 3.5 postcoitum (pc) blastocyst [3, 4]. When ES cells are grown as cell aggregates (embryoid bodies (EBs)) and in the absence of leukemia inhibitory factor (LIF), they differentiate "spontaneously" to ectoderm, endoderm, mesoderm, and their various derivatives [5–7]. Although ES cell *in vitro* differentiation appears to be a promising analytical tool for the study of the factors involved in mammalian development, its logical exploitation has been confounded by the presence of serum in the culture medium. Serum contains a multitude of poorly characterized components which muddle the analysis of any specific growth factor response. We have previously demonstrated that a serum-free, chemically defined medium (CDM) can support ES cell proliferation and differentiation [8]. This CDM–ES model system resembles aspects of mouse development and has many of the advantages of the *X. laevis* animal cap assays.

Previous work using *Xenopus* animal cap assays suggested that neuroectoderm development can occur in the absence of exogenous signaling factors [9–11] or in the presence of antagonists to mesoderm-inducing factors [12–15]. We sought here to ask if a similar control system occurs in mouse ES cells and hence possibly in the mouse *in vivo*.

We present data which, when combined with previous *in vivo* and *in vitro* studies using mouse [8, 16, 17] and *Xenopus* [18–21], indicate that BMP-4 or similar molecules are involved in controlling primitive ectoderm fate during early mouse development and that in the absence of BMP-4 or similar molecules neuroectodermal differentiation occurs. Data presented here support the view that these processes involve cohorts of factors operating antagonistically and synergistically and that ES cell *in vitro* systems can assist in unraveling these interactions.

MATERIAL AND METHODS

ES cell culture and differentiation. CCE ES cells (129/Sv/Ev-derived) were adapted to grow on gelatinized flasks in Dulbecco's modified Eagle's medium supplemented with 15% fetal bovine serum (FBS), 150 μM monothioglycerol, and LIF [22, 23]. For these experiments, CCE cells were grown for a maximum of 20 passages in the absence of feeder cells and were capable of germline transmission before feeder-free adaptation. ES cell lines D3 (129/Sv-derived [5]) and E14.1 (129/Ola-derived [24]) were maintained in the same media but on gamma-irradiated primary embryonic mouse fibroblast feeders. Passages of E14.1 cells used for these experiments were capable of germline transmission.

CDM formulation and ES cell differentiation in CDM were as described in [8], except ES cells were cultured for 30–60 min in CDM with 1000 unit/ml LIF before washing and trypsinization. This re-

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duced any FBS-derived growth factors present and eliminated any detectable Brachyury expression reported previously with CDM alone [8]. After trypsinization, trypsin inhibitor (Sigma, Cat. No. T-6522) was used to neutralize trypsin before cells were resuspended in CDM. To initiate differentiation, 6000 ES cells were seeded per 35-mm bacterial grade dish in 1 ml of CDM; at this cell density with CCE cells, 10–20 EBs/ml formed after 5 days. For the standard CDM differentiation studies, ES cells were trypsinized to single cells and washed completely free of LIF and 5000 cells were seeded into a 35-mm bacterial grade dish in 1 ml of CDM. This would give rise to 10–20 EBs/ml after 5 days.

CDM formulation was as follows: Iscove's modified Dulbecco's medium:Ham's F12, at a 1:1 ratio, supplemented with glutamine as Glutamax-I (Gibco BRL Life Technologies, Inc.); bovine serum albumin (BSA) at a concentration of 5 mg/ml (Boehringer Mannheim, Cat. No. 652 237); a mixture of synthetic lipids at 1× (stock at 100×; Gibco BRL Life Technologies, Inc., Cat. No. 11905015); LIF at 1 unit/ml (Gibco BRL (ESGRO) Cat. No. 13275); transferrin at a final concentration of 150 μg/ml (Boehringer Mannheim, Cat. No. 1073982); monothioglycerol at 450 μM; insulin at 7 μg/ml. In some experiments the BSA component of CDM was replaced with 0.1% w/v polyvinyl alcohol (Sigma P-8136), this is referred to as P-CDM. The use of polyvinyl alcohol makes an almost protein-free, completely chemically defined medium (based on suggestions by Kane [25]). The use of P-CDM gave identical results to CDM (not shown). Many batches of these various reagents have been used without noticeable variation.

Exogenous factors were added at the beginning of the differentiation period. Recombinant human Activin A and BMP-2, -4, -6, and -7 as purified homodimers were obtained as gifts from the Genetics Institute, Inc. (Cambridge, MA). Each batch of factor was titered for maximal mesoderm-mediating activity (data not shown and see [8]). Purified human recombinant follistatin was a gift from the National Hormone and Pituitary Program (Ogden BioServices Corp., Rockville, MD).

RT-PCR. Semiquantitative RT-PCR was performed as described [7, 8], except that cDNA was synthesized using random primers. The resulting cDNA was quantified by PCR using HPRT (hypoxanthine phosphoribosyltransferase) as a cDNA reference standard. A Biometra TRIO-thermoblock thermal cycler was used for all PCRs excepting noggin (see below), operating on a regime of 96°C for 6 s; 50, 55, or 60°C for 15 s; 72°C for 60 s for 31 cycles followed by 72°C for 10 min. PCR were of 15-μl volume, using cDNA derived from an estimated 20 cells for HPRT standardization and 100 cell equivalents for all other genes, see [7, 8]. In some RT-PCRs primers for HPRT and Brachyury were combined, resulting in both targets being amplified simultaneously. Half of the resulting PCR products were gel electrophoresed and Southern blotted onto Gene Screen Plus (Du Pont) followed by hybridization with appropriate probes. Hybridized filters were washed in 0.1× SSC plus 0.1% SDS at 65°C for 2× 20 min [26]. Signals were visualized and quantified relative to HPRT expression using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Most PCR primers were designed using assistance from MacMolly "Tetra" software (Soft Gene GmbH, Germany). Criteria for primer optimization and specificity were as published before [7, 8, 27]. Additional primers used: Alk-3, Accession No. Z23154, 5'TCACCGAAAG CCCAGCTACG and 5'TCACCGAAAG CCCAGCTACG, annealing at 55°C, cDNA product 700 bp; Brachyury, Accession No. X51683, 5'ATGCCAAAGA AAGAAACGAC and 5'AGAGGCTGTA GAACATGATT, annealing at 55°C, cDNA product 836 bp; fyn, Accession No. M27266, 5'CAACCGGGGA AACTGGTTAC and 5'GCTCATGTA CTCCGTGACGA, annealing at 55°C, cDNA product 645 bp; goosecoid, Accession No. M85271, 5'GCACCATCTT CACCGATGAG and 5'AGGAGGATCG CTTCTGTCGT, annealing at 55°C, cDNA 179 bp; Nodal, Accession No. X70514, 5'TCACCGTCCC CTCTGGCGTA and 5'ACTCCTCCCC CACAGGGTTA, annealing at 60°C, cDNA product 773 bp; Noggin, Accession No. U79163, 5'TG-

GCGGCCGC CTTCCCAAGT and 5'AGCCCGGGGG ATCCATC-AAG, PCR conducted on a PTC200 machine (MJ Research, MA) at 96°C for 20 s, 60°C for 15 s, 72°C for 60 s, 30 cycles, cDNA product 365 bp, was cloned and sequenced to verify identity; and pax-6, Accession No. X63963, 5'CAGTCACAGC GGAGTGAATC and 5'CGCTTCAGCT GAAGTCGCAT, annealing at 55°C, cDNA product 658 bp.

Water was used as a negative control. Other PCR primers used were as published [7, 8]. RT-PCR positive controls were for Alk-3, E6.5 egg cylinder [28]; for Brachyury and goosecoid, EBs grown in FCS containing medium for 4 days [7]; for follistatin, day E5.5 decidua [29]; for fyn, E8.5 embryo proper [30]; for pax-6, E11.5 embryo [31]; for Noggin E9.5 day head region (International Publication WO 94/05791); and for Nodal, E7.5 p.c. egg cylinder [32].

RESULTS

Pax-6 Is Expressed by EBs Grown in CDM, in the Absence of Brachyury

ES cell lines CCE, D3, and E14.1 rapidly lose ES cell-specific markers when cultured as EBs in CDM. During this differentiation there is no concomitant increase in markers for mesoderm or mesoderm-derived cell types [8]; as such, we wished to determine ES cell fate under this regime. Published work using *Xenopus*
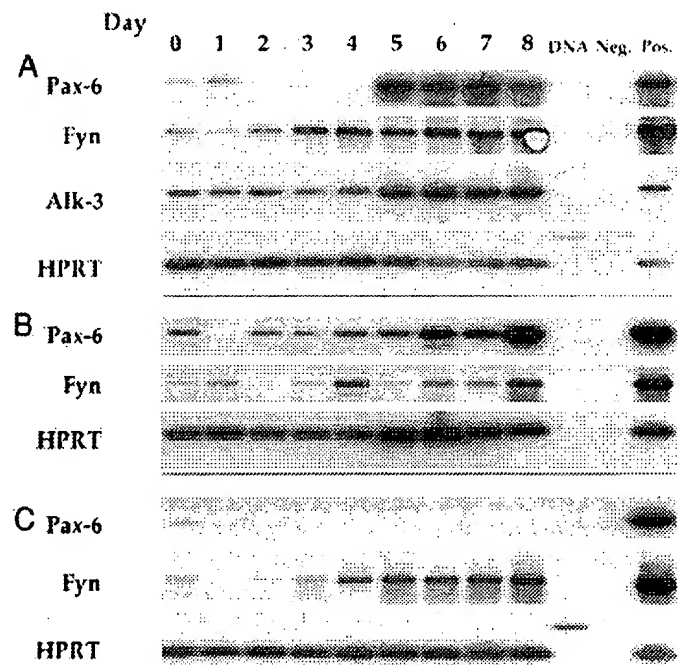

FIG. 1. Time course analysis of pax-6, fyn, and Alk-3 expression in EBs grown in CDM ± BMP-4 or Activin A. Southern blots of RT-PCR analysis on ES–EB grown in CDM ± BMP-4 or Activin A. (A) EBs grown in CDM for 0–8 days, (B) CDM plus Activin A, (C) CDM plus 2.0 ng/ml BMP-4. For HPRT, 20 cell equivalents of cDNA were used per RT-PCR (see [7]); for pax-6, fyn, and Alk-3, 200 cell equivalents. Resulting RT-PCR products were gel electrophoresed and Southern blotted onto Gene Screen Plus (Du Pont) followed by hybridization with appropriate probes. Derived from the linear output of a PhosphorImager (Molecular Dynamics). Controls are listed under Material and Methods.

TABLE 1

Summary of Gene Expression Level

Treatment	Gene	Day when expression >3-fold compared with ES cells	Day of maximal expression level compared with ES cells
CDM alone	Brachyury	Not detected	<1
	Alk-3	~Day 5	>3-fold by day 5
	Pax-6	Day 5	>10-fold by day 5
	Fyn	Day 3	>10-fold by day 6
CDM + Activin A	Brachyury	Day 5 [10]	Not done
	Pax-6	Days 5–6	>8-fold by 6–8 days
	Fyn	Days 4–8	>10-fold by day 8
CDM + BMP-4 (2.0 ng/ml)	Brachyury	Day 3	>20-fold by day 3
	Goosecoid	Days 1–3	>20-fold by day 3
	Nodal	Days 2–3	>20-fold by day 3
	FGF-5	Days 2–3	>20-fold by day 3
	Alk-3	Day 4	>10-fold by day 6
	Pax-6	Not upregulated	<1
	Fyn	Days 4–5	>10-fold by day 8

Note. CCE ES cells were seeded in CDM and harvested from 0 to 8 days for RT-PCR analysis. RT-PCR products were gel electrophoresed, Southern blotted, and hybridized with appropriate probes (see Figs. 1 and 2). Quantification was done using a PhosphorImager; HPRT was used as a cDNA loading standard and the level of gene expression was compared with that found in undifferentiated ES cells. Controls are as listed under Material and Methods.

revealed that neuroectoderm can develop in the absence of mesoderm without exogenous growth factors. We extended this approach to mammals by differentiating ES cells in CDM, in the absence of exogenous factors for 0 to 8 days. As reported previously, there is a rapid decline in Rex-1 and activin βB RNAs, indicative of the loss of ES cell phenotype, but with no consequent increase in Brachyury (see Fig. 3, lane CDM) or goosecoid expression [8, 33]. As a marker for neuroectoderm we examined the expression of the transcription factor pax-6 [31, 34, 35]. Surprisingly, pax-6 RNA was detectable in undifferentiated ES cells (CCE, D3, and E14.1) at low, variable levels by RT-PCR analysis. Upon culturing for 2–3 days in CDM, pax-6 expression became undetectable; however, after 5 days it increased by more than 10-fold over that seen in undifferentiated cells (Fig. 1 and Table 1). Similar results were also obtained with ES cell lines E14.1 and D3 (not shown).

A number of nonexclusive neural markers exist, for example, fyn, a nonreceptor tyrosine kinase, which is first detectable in the developing head-fold region of the E7.5 mouse embryo in cells of both neuroectodermal and nonneuroectodermal origin [30]. Fyn mRNA was detectable at low levels in undifferentiated ES cells. EBs grown in CDM for 3 days showed an increase in fyn abundance by more than 3-fold. By day 8 this increase was greater than 10-fold over that seen in undifferentiated ES cells (Fig. 1 and Table 1). The nonexclusive neural marker N-CAM was also upregulated under these conditions (not shown). Similar de-

velopment was observed when EBs were grown in a variant of CDM, P-CDM (see Material and Methods).

Follistatin mRNA is first detectable *in vivo* in the posterior region of the primitive streak and during *in vitro* differentiation of ES cells in the presence of FBS [29]. Follistatin expression was found at low, variable levels during the time period monitored, 0 to 8 days, for ES/EB differentiation in CDM. Noggin and chordin expression were also analyzed and found to be only just detectable during differentiation in CDM alone (not shown).

EBs Express Pax-6 and Mesoderm Markers When Grown in CDM Plus Activin A

CDM plus Activin A promotes the formation of dorsoanterior-like mesoderm [8]. Using aggregates of *Xenopus* animal cap cells, it has been shown that Activin A induces both mesoderm and neuronal development [36]. We examined the expression of both mesodermal and neuronal makers in EBs grown in CDM plus Activin A. As reported before, mesoderm markers goosecoid and Brachyury were detectable within 5 to 6 days of differentiation [8]. RNA abundance for pax-6 and the nonexclusive neuroectodermal marker fyn was also examined. RNAs for both were upregulated by at least threefold after 4–5 days under these conditions (Fig. 1 and Table 1). Follistatin RNA was also upregulated (Table 2), possibly as the result of mesoderm formation (see [29]). Thus, in the presence of Activin A dorsoanterior mesoderm and neuroectoderm develop simultaneously in EBs.

TABLE 2

Summary of Gene Expression in CCE EBs Differentiated for 5 Days in CDM ± BMP-4 or Activin A

Differentiation conditions	HPRT per 20 cell equivalents	Brachyury per 200 cell equivalents	Follistatin per 200 cell equivalents	Pax-6 per 200 cell equivalents
Undifferentiated ES cells	+	−	−	±[a]
CDM alone	+	−	−[a]	++
CDM plus BMP-4				
8.0 ng/ml	+	−	−	−
6.0 ng/ml	+	−	+	−
4.0 ng/ml	+	−	+++	−
2.0 ng/ml	+	−	+++	−
1.5 ng/ml	+	−	+++	−
1.0 ng/ml	+	−	+	−
0.5 ng/ml	+	+	++	++
0.25 ng/ml	+	+	>+++	+++
0.12 ng/ml	+	−	+	>+++
0.06 ng/ml	+	±	+	++
CDM plus Activin A				
2.0 ng/ml	+	++	>+++	+[a]
Positive controls[b]	+	++	+	++

Note. CCE ES cells were seeded in CDM, CDM plus BMP-4 at 0.06–8.0 ng/ml, or Activin A at 2.0 ng/ml. After 5 days EBs were harvested for RT-PCR analysis. cDNAs were equalized using HPRT as a standard. For HPRT, 20 cell equivalents of cDNA were used per RT-PCR (see [7]); for Brachyury, follistatin, and pax-6, 200 cell equivalents. The results are from a single representative experiment. Water was used as a negative control. Scoring was by eye based on ethidium bromide staining of the gel; −, not detected; ±, faint band visible; + to +++, positive to very strong signal.
[a] Variable at this time point between experiments.
[b] cDNAs used for positive controls are listed under Material and Methods.

Pax-6 Expression Is Inversely Dependent upon BMP-4 Concentration

It has been proposed that BMP-4 counteracts neural development in *Xenopus* [21, 37–40]. If an analogous system exists in mammals it would be expected that BMP-4 would override neuroectodermal formation in EBs.

Upon the addition of BMP-4 at 2.0 ng/ml to ES cells in CDM, the low levels of pax-6 expression seen in undifferentiated ES cells became undetectable by 48 h of culture and remained so for the time period monitored (8 days) (Fig. 1). In contrast to pax-6, the nonexclusive neuroectodermal marker, fyn, was readily detectable under these conditions at 4 to 5 days. Fyn expression was similar to that observed with CDM alone or CDM plus Activin A (Fig. 1). The concentration of BMP-4 used in these studies has been previously shown to give a rapid upregulation of mesodermal markers (see below). When, however, we examined the effect of different BMP-4 concentrations on pax-6 expression, a more complex picture emerged. Previously, we have shown that as the concentration of BMP-4 was reduced mesoderm development showed an increasing lag time in being expressed (see [8]). Pax-6 expression showed an inverse relationship to BMP-4 concentration (Table 2). Follistatin expression was also influenced by BMP-4 concentration (Table 2).

BMP-Mediated Differentiation in EBs

To compare mesoderm formation in EBs to that *in vivo*, we monitored the expression of genes known to be associated with primitive streak formation and the process of gastrulation. For these experiments ES cells were grown for 0 to 8 days in CDM plus 2.0 ng/ml BMP-4. This concentration of BMP-4 promotes rapid mesoderm formation [8].

The genes monitored included Brachyury, expression of which is first detected in the developing primitive streak and the newly emerging mesoderm [41]; goosecoid, first expressed in the E6–E6.5 egg cylinder as a patch of cells at the anterior end of the newly developing primitive streak [42]; Nodal, detectable in ES cells and in the early egg cylinder [8, 32]; and fibroblast growth factor-5 (fgf-5), which is found in the embryonic ectoderm just prior to gastrulation [43]. Compared with undifferentiated ES cells, Brachyury, goosecoid, Nodal, and fgf-5 RNAs all showed a pulse of expression greater than 20-fold at days 2–3 of ES–EB development. Over the subsequent 24–48 h of EB differentiation expression diminished to low or undetectable levels. The striking simultaneous upregulation of these genes in EBs is similar, at least at the molecular level, to primitive streak formation in mouse.

The BMP type I receptor, Alk-3, encodes a serine/threonine kinase capable of binding BMP-2, -4, and -7

with different affinities. It is highly expressed in the egg cylinder and is essential for mesoderm development [17, 28, 44]. We found that Alk-3 was expressed at low levels in undifferentiated ES cells. During ES cell differentiation in CDM plus BMP-4, Alk-3 abundance increased, reaching more than threefold that seen in ES cells by day 3, continuing to rise thereafter (Fig. 2 and Table 1). This suggests that both ES cells and their differentiated progeny are capable of binding BMP-2, -4, and -7 [28, 44] and are thus competent to respond to all these factors. Other BMPs which may be involved in mesoderm formation include BMP-6 and -7. BMP-7 has been reported as a weak inducer of mesoderm formation in *Xenopus* [45] and has also been shown to cause differentiation of the human EC cell line NTERA2 [46]. Furthermore, both BMP-6 and BMP-7 are present in the embryo at E6.5 [47, 48]. To test if these factors could have a possible role in mesoderm formation we cultured EBs in CDM plus BMP-6 (10 ng/ml) or BMP-7 (2.0 ng/ml) for 5 days. Multiplex RT-PCR analysis revealed a marked increase in Brachyury expression (vs HPRT) compared with undifferentiated ES cells or ES cells grown in CDM alone (Fig. 3). Goosecoid expression also increased in the presence of these factors (not shown). This indicates that BMP-6 and -7 are capable of mediating mesoderm development *in vitro*.

DISCUSSION

ES cells originate from the ICM of the 3.5 day pc blastocyst and retain many similarities to these cells in culture. They can be maintained as undifferentiated
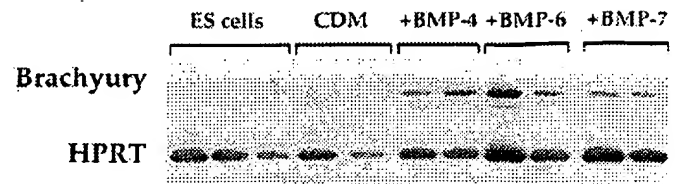

FIG. 3. Multiplex RT-PCR examining the expression of HPRT relative to Brachyury. Multiplex RT-PCR analysis using primers for both HPRT and Brachyury, followed by Southern blotting and hybridization as outlined under Material and Methods. The results of undifferentiated ES cells (200, 60, and 20 cell equivalents) and EBs grown for 5 days in CDM alone, CDM plus 0.25 ng/ml BMP-4, CDM plus 10 ng/ml BMP-6, and CDM plus 2.0 ng/ml BMP-7, all at 60 and 20 cell equivalents are shown. Derived from the linear output of a PhosphorImager.
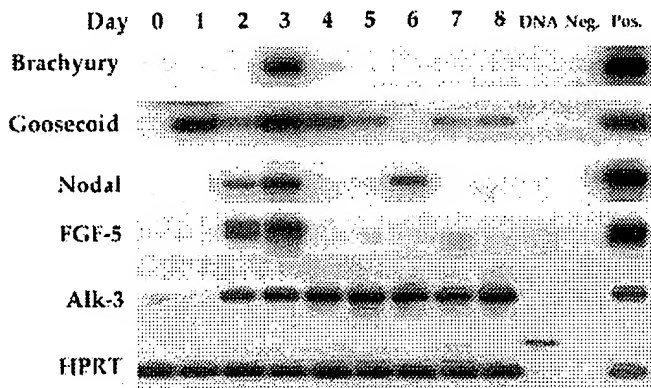

FIG. 2. Time course analysis of genes related to mesoderm formation in ES–EB grown in CDM plus 2.0 ng/ml BMP-4. RT-PCR products were gel electrophoresed, Southern blotted, and hybridized as outlined under Material and Methods. cDNA loading was relative to HPRT using 20 cell equivalents of cDNA per RT-PCR (see [7]), for Brachyury, 200 cell equivalents. Signals were visualized and quantified relative to the expression level in undifferentiated (day 0) ES cells. Derived from the linear output of a PhosphorImager. Negative control used water, positive controls are listed under Materials and Methods.

cell lines in the presence of LIF and FBS; however, upon removal of LIF they differentiate rapidly, forming predominantly endoderm, mesoderm, and, to a lesser extent, hematopoietic cells. These avenues of differentiation are facilitated by the absence of LIF and by the presence of undefined factors in FBS [7, 8].

In contrast, when ES cells are grown in a serum-free medium (CDM) they rapidly lose their ES cell phenotype without forming mesoderm [8] and develop, together with other cell types, neuroectoderm. This is analogous to the *Xenopus in vitro* animal cap assays, in which prospective ectoderm presumes a neuronal fate upon blocking of BMP-4 or similar molecules [21, 38, 40] or on the disruption of activin-responsive pathways [10, 11]. Under the culture conditions described here, ES cells rapidly form cell aggregates, thus it is difficult to assess if neuroectoderm differentiation is cell autonomous or involves cell–cell interactions. It is possible that neuroectoderm development and/or cell survival is motivated/supported by factors synthesized by ES cells or from the newly forming endoderm which develops around the EBs. However, data derived from studies using disaggregated *Xenopus* blastula or early gastrula ectoderm followed by reaggregation suggest that neuronal commitment can occur as single cells [49, 50]. Thus vertebrate neuroectodermal commitment *can be* a permissive or possibly a cell-autonomous "default state" for ectoderm, in which absence or sequestration of mesoderm-inducing factors from regions of the embryo facilitates an ectoderm to neuroectoderm transition. Previous reports have shown that both embryonal carcinoma (EC) and ES cells can at a low frequency develop into neuronal-like cells upon treatment with retinoic acid and cell aggregation (after >10 days with batch-selected FBS) [51, 52]. A putative pathway for this is raised by the observation that retinoic acid treatment of EC cells leads to a marked increase in follistatin expression [53] and possibly other anti-TGFβs, e.g., chordin and Noggin. Further, retinoic acid blocks the expression of the mesodermal genes

Brachyury, cardiac actin, and ζ-globin [54]. The neutralization activity of activin or similar mesoderm-inducing substances in FBS would thus facilitate neuroectoderm development over that of mesoderm. As EBs grown in CDM do not upregulate follistatin, chordin, or Noggin (M.V.W., data not shown), these factors are not essential for neuroectoderm formation in the absence of mesoderm. Additionally, when human follistatin is added to CDM there is no detectable effect on EB differentiation (M.V.W., data not shown).

Dorsoanterior-like mesoderm and neuroectoderm develop when EBs are treated with Activin A in CDM [8]. This observation is concordant with data derived from *Xenopus* animal cap assays, in which both mesoderm and general neuronal makers are detectable upon Activin A treatment [36]. Neuroectoderm development under these conditions may be secondary and possibly dependent upon the formation of dorsoanterior mesoderm, e.g., possibly by supplying survival factors.

BMP-4 in CDM mediates the formation of posterior and/or extraembryonic-like mesoderm in EBs in a concentration-dependent manner [8]. We show here that this process appears as a synchronized pulse of gene expression (including Brachyury, goosecoid, and Nodal), reminiscent of primitive streak formation *in vivo*. Such a pulse suggests that once mesoderm triggering has occurred there is a swift recruitment of ES-derived primitive ectoderm-like cells to mesoderm. As BMP-4 can cause upregulation of itself and of BMP-2, the initiation of a positive feedback would account for this effect [8]. Further, the observation that BMP-6 and -7 can facilitate mesoderm formation is in agreement with the concept that this closely related group of BMPs is involved in mesoderm development in mouse.

Mesoderm formation almost certainly involves the interaction of many factors, with probably no single factor acting as a prime instigator of all mesoderm formation. For example, anterior mesoderm still forms (poorly) in BMP-4 null-mutant embryos [55]. However it is likely that BMPs are the key to mesoderm formation as Alk-3 null-mutant embryos fail to develop any mesoderm [17].

Conclusion

From the data presented here and from other studies, it is clear that early embryonic development involves many localized transient combinations of factors. Such developmental networks operate as integrated units providing a high degree of developmental resilience and compensation [56]. These interwoven networks are also difficult to resolve. ES cell *in vitro* differentiation approaches do not represent a simplification of these mechanisms; however, they do offer an opportunity to reduce such processes to experimentally amenable units. Further, data presented here clearly show that many

aspects of both neuroectodermal and mesodermal formation observed in *Xenopus* are also relevant to mouse.

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